The Formation of Competent Barrier Lipids in Reconstructed Human Epidermis Requires the Presence of Vitamin C

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Our analysis of epidermal lipids revealed that (glucosyl)ceramide profiles in various human skin equivalents are different from those of native tissue. The main difference is the reduced content in skin equivalents of ceramides 4–7 and especially the very low content of the most polar ceramides 6 and 7, which contain hydroxylated sphingoid base and/or fatty acid. To facilitate hydroxylation, the culture medium was supplemented with vitamins C and E. Although in vitamin E-supplemented medium lipogenesis was not affected, in vitamin C-supplemented medium the content of glucosylceramides and of ceramides 6 and 7 was markedly increased, both in the presence and absence of serum and irrespective the substrate used (inert or natural, populated or not with fibroblasts). The improvement of the lipid profile was accompanied by a marked improvement of the barrier formation as judged from extensive production of lamellar bodies, their complete extrusion at the stratum granulosum/stratum corneum interface, and the formation of multiple broad lipid lamellar structures in the intercorneocyte space. The presence of well-ordered lipid lamellar phases was confirmed by small-angle x-ray diffraction. Some differences between native and reconstructed epidermis, however, were noticed. Although the long-range lipid lamellar phase was present in both the native and the reconstructed epidermis, the short lamellar phase was present only in native tissue. It remains to be established whether these differences can be ascribed to small differences in relative amounts of individual ceramides, to differences in fatty acid profiles, or to differences in cholesterol sulfate, pH, or calcium gradients. The results indicate the key role vitamin C plays in the formation of stratum corneum barrier lipids. *Key words: stratum corneum lipid organization/composition/ultrastructure/small-angle x-ray diffraction. J Invest Dermatol 109:348–355, 1997*

The stratum corneum (SC), a protective barrier against the transepidermal water loss and the penetration of various substances from the environment, is a highly specialized tissue consisting of cornified cells surrounded by extracellular lipid lamellae that consist predominantly of ceramides, cholesterol, and free fatty acids (reviewed in Schhrer and Elias, 1991; Wertz and Downing, 1991) present in approximately equimolar ratios (Man *et al*, 1993). The epidermis is a metabolically active tissue synthesizing all lipids required for the barrier formation. Especially unique for the epidermis is the synthesis of large amounts of glucosylceramides and ceramides.

Although in recent years various human skin recombinants have been developed that show a close resemblance with native epidermis, their SC barrier function is impaired (reviewed in Ponec, 1992). Analysis of epidermal lipid composition of human epidermis reconstructed on de-epidermized dermis (DED) revealed that the content of glucosylceramides, ceramides, and free fatty acids is much lower than that of native tissue (Ponec *et al*, 1988). In addition, the ceramide profile deviated from that found in native epidermis, the main differences being the very low content of ceramides 6 and 7, which represent a dominant ceramide fraction (at least 20% of total ceramides; Long *et al*, 1985). At present it is unclear whether the observed divergences in lipid profile may explain the impaired organization of the SC lipids as observed in electron microscopic (Fartasch and Ponec, 1994) and x-ray diffraction (Bouwstra *et al*, 1995) studies.

Various attempts have been made to improve the quality of the barrier function such as supplementation of medium with vitamin D_3 (Mak *et al*, 1991) and essential fatty acids (Ponec *et al*, 1990, Boyce and Williams, 1993), reducing the relative humidity (Mak *et al*, 1991) or the temperature (Ponec *et al*, 1997), or the use of serum-free medium (Rosdy and Clauss, 1990; Gibbs *et al*, in press). Although some of these modulations led to improvements of the lipid profile such as normalization of triglyceride content and an increase in relative amounts of free fatty acids and total ceramides

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Abbreviations: DED, de-epidermized dermis; RE-DED, epidermis reconstructed on DED; CSS, cultured skin substitute; SG, stratum granulosum; SC, stratum corneum; LB, lamellar body; GSL, glucosphingolipids; SAXD, small angle x-ray diffraction.

(Ponec *et al*, 1997; Gibbs *et al*, in press), the content of glucosylceramides still remained low, and the proportions of ceramides 4–7 were reduced, the largest reduction occurring in ceramides 6 and 7. The constituents of ceramides 6 and 7 are hydroxylated derivatives of a sphingoid base: ceramide 6 contains phytosphingosine and ceramide 7 contains 6-hydroxysphingosine (6-hydroxy-4-sphingenine; Long *et al*, 1985, Robson *et al*, 1994). Furthermore, both ceramides contain an α-hydroxy acid. It is unclear why the synthesis of these most polar ceramides is inhibited most, because ceramide 3, which is abundantly synthesized in the reconstructed epidermis, contains phytosphingosine as a base, and ceramides 4 and 5, which contain amide-linked α-hydroxy acids, are also present in small amounts. In addition, ceramide 4 has recently been shown to contain the same sphingoid base as does ceramide 7 (Robson *et al*, 1994).

Little is known about the enzymes and cofactors involved in the hydroxylation of sphingoid bases and fatty acids, but one can speculate that in analogy to other hydroxylation processes [such as the hydroxylation of proline in collagen synthesis, the synthesis of epinephrine from tyrosine, and the initial 7α-hydroxylase step in bile acid formation (Mayes, 1990)], the presence of ascorbate as a cofactor may be required. In addition, the synthesis of phytosphingosine, hydroxysphingosine, and α-hydroxy acids requires the presence of operative electron-transport reactions. In mammalian tissues, most of the hydroxylation steps that are carried out by the cytochrome P-450 family (Estabrook, 1978) require the presence of NADPH dehydrogenase and oxygen. In addition, α-hydroxylation of fatty acids has been demonstrated to be catalyzed by a monooxygenase requiring NADPH and oxygen (Shigematsu *et al*, 1990). Because lipid peroxidation occurs during these reactions, one can assume that for proper functioning of the involved enzymes, the presence of an operational anti-oxidant mechanism might be required. Various anti-oxidant systems have been identified in the skin (reviewed in Fuchs *et al*, 1991) in which α-tocopherol (vitamin E) is the major lipophilic anti-oxidant inhibiting the lipid peroxidation, and ascorbate (vitamin C) is the major hydrophilic anti-oxidant. In spite of the fact that some human skin equivalents have been generated in medium supplemented with vitamin E, the content of ceramides 6 and 7 were extremely low (Gibbs *et al*, in press; Vičanová *et al*, in press). Because ascorbate is involved in the regeneration of vitamin E, the presence of both vitamins might be required for the optimal course of electron-transport reactions. Therefore, the potential role of vitamins C and E on the epidermal lipogenesis was examined.

MATERIALS AND METHODS

Cell Culture Cultures of normal human epidermal keratinocytes and dermal fibroblasts were established as described earlier (Ponec *et al*, 1977, 1981). Fibroblast-populated collagen gels (containing 1×10^5 cells per ml) were prepared as described by Smola *et al* (1993) and DED were prepared as described earlier (Ponec *et al*, 1988). Reconstructed epidermis was generated by seeding of 2×10^5 second-passage human keratinocytes per cm^2 onto DED (Ponec *et al*, 1988), fibroblast-populated collagen matrix (Smola *et al*, 1993), or inert filter (Costar, Cambridge, MA). The following culture protocol was used: The cells were cultured under submerged conditions overnight when seeded on fibroblast-populated collagen matrices, for 3 or 5 d when seeded on DED or on a filter, respectively. Thereafter, the cultures were lifted to the air-liquid interface and cultured for additional 14 d. When serum-containing medium was used, the cultures were fed throughout the whole culture period with a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 5% HyClone serum (Hyclone Laboratories, Inc., Logan, UT), 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin. At the time of exposure to the air, the same medium was used with additional 1 ng EGF per ml. For cultures grown in serum-free medium, the cells were first incubated overnight in culture medium consisting of a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 1% HyClone serum (Hyclone), 0.5 μM hydrocortisone, 1 μM isoproterenol, and 0.1 μM insulin. The next day the cultures on fibroblast-populated collagen matrices were lifted to the air-liquid interface and cultured for 14 d in serum-free medium containing the same supplements with the addition of 10 μM carnitine, 1 mM serine, and a mixture of fatty acids (25 μM C16:0, 25 μM C18:1, 15 μM C18:2, 7 μM C20:4; Boyce and Williams, 1993). Cells seeded on DED or on filters were cultured under submerged conditions for additional 2 d (DED) or 4 d (filter) in the same medium but supplemented with 1% serum. When lifted to the air-liquid interface, the cultures were grown for 14 d in serum-free medium with supplements as above and additional 1 ng EGF per ml. When the effects of vitamin C or vitamin E were examined, the medium was supplemented from the onset of the culture with 50 μg ascorbic acid per ml and/or 1 μM α-tocopherol. All additives were purchased from Sigma (St. Louis, MO). The medium was refreshed three times per week. The cultures were grown either at 37°C or 33°C.

Histology Cultures were fixed in 4% buffered formaldehyde and processed for embedding in paraffin. Vertical sections were stained with hematoxylin and eosin.

Electron Microscopy Specimens were processed for electron microscopy as described earlier (van der Meulen *et al*, 1996). Briefly, cultures were fixed in 2% paraformaldehyde/2% glutaraldehyde and then post-fixed in 1% osmium tetroxide. For optimal visualization of SC lipid bilayers, some samples received additional post-fixation with aqueous 0.5% ruthenium tetroxide. After fixation, the specimens were dehydrated in graded ethanol up to 70% and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead hydroxide.

Lipid Extraction and Separation Tissue specimens were heated for 1 min at 60°C to separate the epidermis from the dermal substrates. Epidermal lipids were extracted by using the method of Bligh and Dyer (1959), dissolved in chloroform/methanol, 2:1 (vol/vol), and stored at −20°C under nitrogen until use. The extracted lipids were separated by 1-dimensional high-performance thin-layer chromatography, as described earlier (Ponec and Weerheim, 1990). For quantification, authentic standards (Sigma) were run in parallel. The quantification was performed after charring with a photodensitometer with automatic peak integration (Desaga, Heidelberg, Germany). For each culture condition, at least three independent experiments ($n \geq 3$) were performed. In each experiment two parallel cultures were used for analysis. For comparison, the lipid profile was analyzed in the following skin equivalents: living skin equivalent (LSE Organogenesis, Cambridge, MA; Parenteau *et al*, 1991), EpiDerm (MatTek Corp., Ashland, MA), Skinethic (kind gift from M. Rosdy; Rosdy and Clauss, 1990), cultured skin substitute (CSS, a kind gift from S. Boyce; Boyce and Williams, 1993). For identification of individual ceramide fractions, the ceramides isolated from the pig SC were used (kindly provided by P. Wertz).

Isolation of SC To separate epidermis from the underlying tissue, the skin equivalents were first incubated for 2 h in 0.1% trypsin in phosphate-buffered saline (PBS). Thereafter the tissue was washed with PBS and the trypsinization was stopped by a short incubation of tissue in PBS containing soybean trypsin inhibitor (10 mg per ml). Subsequently, the epidermis was washed several times with PBS and incubated for 2 h in a 0.01% proteinase K in PBS (Bowser and White, 1985). After extensive washing with PBS and with distilled water, SC was dried under vacuum and stored under nitrogen in the dark until use. Before use the SC was hydrated for 24 h over a 27% NaBr solution, which resulted in an approximate hydration level of 20% [(weight hydrated SC − weight dry SC)/weight hydrated SC].

Small-Angle X-Ray Scattering (SAXD) All measurements were carried out at the Synchrotron Radiation Source at Daresbury Laboratory (Barrington, UK) using station 8.2. This station has been built as part of a NWO/SERC agreement. The small-angle camera was connected with a position-sensitive multiwire quadrant detector. The sample-to-detector distance was set to 1.40 m. A more detailed description of the experimental set up has been given elsewhere (Bouwstra *et al*, 1991).

The SC, approximately 3 mg in weight, was put randomly in a specially designed sample cell with two mica windows. The temperature of this sample cell could be adjusted between 25°C and 120°C. All samples were measured for a period of 15 min. Checks for the appearance of radiation damage were performed but proved to be negative.

The scattering intensities have been plotted as a function of the scattering vector Q defined as $Q = (4\pi \sin \theta)/\lambda$, in which λ and θ are the wavelength and scattering angle, respectively. The position of the diffraction peaks is directly related to the repeat distance of the molecular structure, as described by Bragg's law, $2d \sin \theta = n\lambda$, in which n is the order of the diffraction peak. For a lamellar structure, the various peaks are located at equal interpeak distances, $Q_n = 2n\pi/d$, Q_n being the position of the nth order peak.

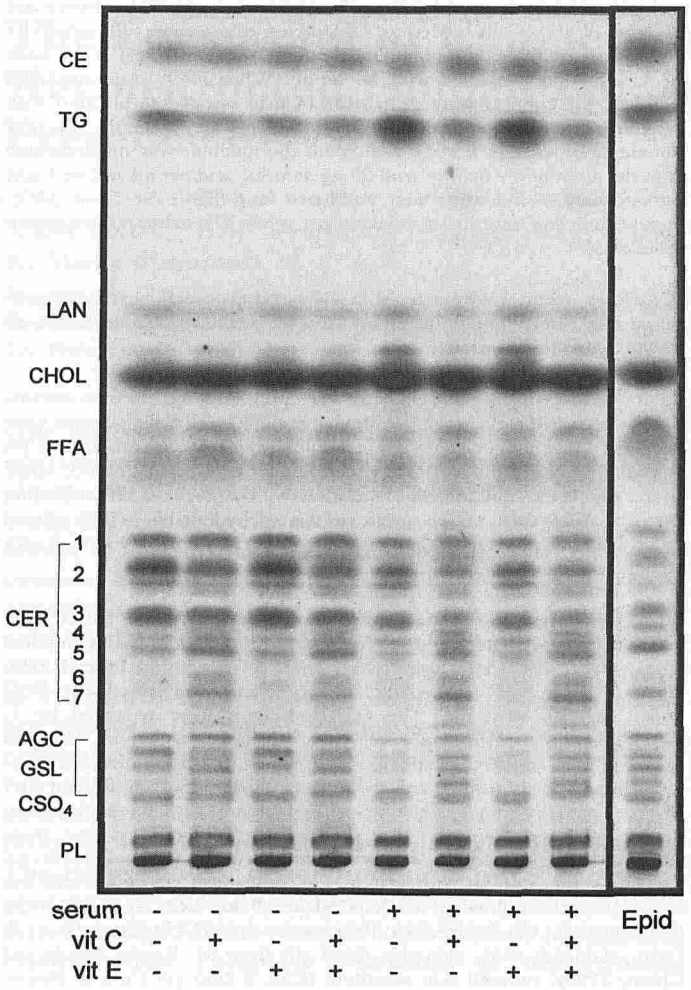

Figure 1. Vitamin C but not vitamin E normalizes epidermal lipid profiles in reconstructed epidermis. Keratinocytes were seeded on DED and grown for 14 d at 37°C at the air-liquid interface in serum-containing (+) or serum-free medium (−) in the absence (−) or presence (+) of vitamin C and E. Thereafter, the epidermal lipids were extracted and subjected to HPTLC using the 'ceramide development system' (Ponec and Weerheim, 1990). For comparison, the lipid profile in native epidermis (Epid) is also shown; 20 μl of total epidermal lipid extracts of individual samples were applied on the HPTLC plate. PL, Phospholipids; CSO_4, cholesterol sulfate; GSL, glucosphingolipids; AGC, acylglucosylceramides; CER, ceramides (1–7); FFA, free fatty acids; CHOL, cholesterol; LAN, lanosterol; TG, triglycerides: CE, cholesterol esters.

RESULTS

Lipid Composition and Ceramide Profile in Reconstructed Epidermis Normalizes in the Presence of Vitamin C Because our recent observations revealed that epidermal growth factor is responsible for high triglyceride synthesis (Ponec *et al*, 1997), the epidermal growth factor concentration in the culture medium at the time of lifting of the culture to the air was reduced from the previously used 10 ng per ml to 1 ng per ml. Because serum components also appeared to affect the lipogenesis (Gibbs *et al*, in press), in the current study we compared lipid profiles in epidermis reconstructed in serum-free and serum-containing media. As shown in **Fig 1**, in keratinocytes cultured in serum-containing medium, the triglyceride content is increased and the glucosphingolipid (GSL) content is decreased. Irrespective of the presence or absence of serum, GSL and ceramide profiles differed from those in native epidermis. Similar observations have been made with various other skin equivalents **(Table I)**. These skin equivalents were generated either in serum-containing [LSE or epidermis reconstructed on DED (RE-DED)] or serum-free medium (EpiDerm, Skinethic, CSS, and recently also RE-DED). In the presence of vitamin E, the lipid profile remained unchanged irrespective the presence or absence of serum **(Fig 1)**. Similar observations have been made with CSS, which is generated in medium supplemented with vitamin E (Boyce and Williams, 1993; **Table I**). In contrast, the supplementation of medium with vitamin C was accompanied with profound changes in the epidermal lipid profile: both the GSL and the ceramide profiles were normalized. Four different GSL fractions (including acylglucosylceramide) and ceramides 6 and 7 were synthesized in significant amounts *in vitro*. This was observed irrespective whether the serum-free or serum-containing medium was used. Upon additional supplementation with vitamin E, the lipid profile remained unchanged **(Fig 1)**.

To examine whether the dermal substrate used for the reconstruction of the epidermis can affect the lipogenesis, the epidermis was generated in serum-free medium and in the presence of vitamin C and E on the following substrates: DED, inert filter, fibroblast-populated collagen matrix, or DED populated with fibroblasts. As shown in **Table II**, under all conditions, the overall lipid profiles were similar and close to those in native epidermis. Also, the relative amount of ceramides was similar to that in native tissue (compare **Tables III** and **I**).

Vitamin C Improves the Epidermal Morphology and the SC Ultrastructure As shown in **Fig 2**, fully differentiating epidermis was developed when keratinocytes were grown in serum-free medium supplemented with vitamin C and E with DED, inert filter, or fibroblast-populated collagen matrix as substrates. All epidermal strata including stratum basale, spinosum, granulosum (SG), and corneum (SC) were present. Electron microscopic investigations of

Table I. Low Content of Polar Ceramides in Various Skin Substitutes[a]

Ceramide	RE-DED[b]	RE-DED[c]	EpiDerm[d]	Skinethic[e]	LSE[d]	CSS[f]	Native epidermis[g]
1	15.6 ± 2.0	14.8 ± 1.1	12.8 ± 1.3	14.9 ± 2.2	15.0 ± 1.9	15.8 ± 2.6	10.2 ± 1.8
2	47.1 ± 1.4	43.8 ± 2.5	48.4 ± 1.5	52.8 ± 1.3	53.0 ± 2.4	57.9 ± 1.7	22.1 ± 0.2
3	20.7 ± 1.6	25.8 ± 0.9	23.2 ± 1.3	28.4 ± 1.1	15.3 ± 0.9	13.8 ± 0.3	21.9 ± 1.6
4	5.5 ± 1.0	4.4 ± 1.1	8.7 ± 0.4	5.9 ± 1.0	5.8 ± 0.9	6.2 ± 0.2	6.9 ± 1.3
5	8.1 ± 1.6	8.1 ± 1.7	3.7 ± 2.2	5.8 ± 1.6	7.5 ± 0.4	6.3 ± 0.4	16.7 ± 1.6
6	3.0 ± 0.5	3.1 ± 1.5	3.2 ± 1.8	2.2 ± 0.6	3.4 ± 0.3	0	7.5 ± 1.5
7	0	0	0	0	0	0	14.7 ± 0.8

[a] All cultures were grown for about 14 days at the air-liquid interface. The epidermis was harvested, and the epidermal lipids were extracted, separated by high-performance thin layer chromatography, and quantified after charring. Data are expressed as weight percents of total ceramides (means ± SD).
[b] RE-DED was generated by culturing of keratinocytes in serum-containing medium (n = 5).
[c] RE-DED was generated by culturing of keratinocytes in serum-free medium (n = 4).
[d] Commercially available skin substitutes EpiDerm (n = 3) and LSE (n = 3) were used.
[e] Skinethic was kindly provided by M. Rosdy (n = 6).
[f] CSS was kindly provided by S. Boyce (n = 3).
[g] Native epidermis was isolated from freshly excised breast skin (n = 8).

Table II. Normalization of Lipid Composition in Reconstructed Epidermis Requires the Presence of Vitamin C[a]

Lipid Fraction	Native Epidermis	Epidermis reconstructed on		
		DED	Filter	Collagen Matrix
Phospholipids	36.5 ± 4.1[b]	36.9 ± 5.2	39.7 ± 3.9	38.6 ± 4.3
Sphingomyelin	8.9 ± 1.6	7.4 ± 2.2	7.7 ± 1.8	6.1 ± 0.8
Phosphatidylcholine	11.2 ± 0.8	11.2 ± 1.2	12.1 ± 2.2	13.3 ± 1.9
Phosphatidylserine	3.9 ± 0.3	3.8 ± 0.8	4.0 ± 1.1	4.6 ± 0.6
Phosphatidylinositol	2.2 ± 0.8	3.5 ± 1.3	4.3 ± 1.3	3.1 ± 0.2
Phosphatidylethanolamine	10.3 ± 0.8	10.8 ± 1.4	11.6 ± 0.9	11.5 ± 2.2
Cholesterol sulfate	5.0 ± 1.6	4.9 ± 2.8	3.5 ± 1.4	4.3 ± 1.1
Glucosphingolipids	5.0 ± 0.4	4.0 ± 1.8	2.5 ± 0.9	3.8 ± 2.2
Ceramides	12.1 ± 1.8	15.8 ± 2.7	14.6 ± 2.2	15.4 ± 2.8
Free fatty acids	7.8 ± 1.2	5.2 ± 1.6	7.0 ± 2.3	6.3 ± 2.9
Cholesterol	17.7 ± 3.2	20.3 ± 4.4	23.3 ± 2.2	18.8 ± 3.6
Lanosterol	—	0.7 ± 0.3	0.8 ± 0.2	0.6 ± 0.1
Triglycerides	8.9 ± 3.7	9.7 ± 3.2	10.6 ± 2.1	9.8 ± 1.2
Cholesterol esters	7.0 ± 0.4	2.5 ± 1.2	5.0 ± 1.1	2.4 ± 0.6

[a] Keratinocytes were seeded on DED, inert filter, or fibroblast-populated collagen matrix and grown for 14 days at the air-liquid interface in serum-free medium supplemented with vitamins C and E. Thereafter, the epidermal lipids were extracted, subjected to high-performance thin layer chromatography, and quantified.
[b] Data are expressed as weight of total lipids percent (mean ± SD; n = 3).

these cultures revealed the presence of stellate keratohyalin granules **(Fig 3*a*)** and numerous lamellar bodies (LBs) in SG **(Fig 3*b*)**. LBs were extruded at the SG/SC interface **(Fig 3*c*)** and found in saccular extracellular domains. As in native epidermis (Fartasch *et al*, 1993), the exocytosis of LB lipids into the intercellular space was completed at the SG/SC interface with subsequent processing into complete lipid lamellae adjacent to the cornified envelope with the electron-dense lipid envelope at the periphery of the corneocyte. Already in the uppermost part of the saccular spaces lipid lamellae were appearing. The periphery of the saccular domains appeared to be connected to the adjacent desmosomal plugs **(Fig 3*c*)**. The intercellular space of the SC was abundantly filled with multilayered lamellar lipid structures **(Fig 4)** that showed the characteristic alternating electron-dense and electron-lucent pattern with repeating pattern of lucent bands with a broad-narrow-broad configuration. No remnants of LBs have been found in the intracorneocyte space. Extensive deposition of mature lipid bilayers has been found throughout the intercellular domains of the SC **(Fig 4*a*)**. Lipid lamellae were inserting into desmosomal plugs, which underwent transformation similar to that in native epidermis (Fartasch *et al*, 1993). The edges of these desmosomal plugs were first round **(Fig 4*a*)** and with progression of degradation they show pointing and have opaque appearance **(Fig 4*b*)**. In the upper SC layers, remnants of the desmosomal plugs with pointed edges surrounded with multilayered lipid lamellar structure can be found **(Fig 4*c,d*)**. These results indicate that the sequences of ultrastructural maturation in reconstructed and native epidermis are similar.

Vitamin C Supplementation Leads to a Marked Improvement of the SC Lipid Organization SAXD profiles of SC isolated from the epidermis reconstructed in serum-free medium supplemented with vitamins C and E were similar regardless the substrate and temperature (37°C or 33°C) used. In all cases three strong reflections were observed at approximately Q = 0.5, 1.0, and 1.6 nm^{-1} and one weak reflection at 2.1 nm^{-1} corresponding to the spacing of 12, 6, 4, and 3 nm, respectively. These reflections are most probably the first, second, third, and fourth order of a lamellar phase with a periodicity of approximately 12 nm. Depending on the substrate used and temperature conditions, the periodicity varied between 11.5 and 12 nm. An example of a SAXD diffraction profile of SC isolated from epidermis reconstructed on an inert filter is shown in **Fig 5**. In a previous study we found that in SC isolated from epidermis reconstructed in serum-containing medium a prominent peak located at Q = 1.87 nm^{-1} (3.35-nm spacing) was often present, indicating the presence of phase-separated polycrystalline cholesterol (Bouwstra *et al*, 1995). In the current study, we noted the presence of only a very weak peak at Q = 1.87 nm^{-1}, indicating that the amount of polycrystalline cholesterol was very low, as was observed as well in native human SC. When the temperature-induced lipid-phase changes were examined, the temperature was raised from 25°C to 95°C with a heating rate of 2°C per min, and each minute the diffraction data were collected. A typical example of temperature-induced changes in SAXD profile of SC isolated from the reconstructed epidermis is shown in **Fig 6**. By raising the temperature up to 60°C, no changes in long-range ordering were observed, as was observed with native SC. In the temperature range between 61°C and 67°C, the diffraction peaks based on the 12-nm phase weakened and finally disappeared. In the same temperature region, a new 4.6-nm peak appeared. In native SC the appearance of a new phase in this temperature region has never been observed (Bouwstra *et al*, 1991). A similar peak, however, was detected in the diffraction patterns obtained with lipid mixtures prepared from ceramides and cholesterol (Bouwstra *et al*, 1996).

DISCUSSION

The ultimate goal in the development of skin substitutes is the generation of an epidermis with a SC exhibiting the same barrier function as the native skin. Because the main barrier for penetration

Table III. Vitamin C Induces Marked Improvement of Ceramide Profile in Reconstructed Epidermis[a]

Substrate	Ceramide (% of total)[b]						
	1	2	3	4	5	6	7
DED	12.9 ± 1.1[b]	27.3 ± 2.3	20.1 ± 1.8	7.0 ± 0.6	12.7 ± 2.8	6.4 ± 1.4	13.7 ± 0.8
Filter	11.2 ± 1.4	30.1 ± 3.1	21.2 ± 0.9	6.5 ± 1.5	10.0 ± 2.0	8.2 ± 0.9	9.8 ± 1.1
Collagen matrix	16.3 ± 2.2	26.3 ± 1.3	17.7 ± 1.1	7.0 ± 1.0	12.9 ± 1.6	8.1 ± 0.6	11.8 ± 2.2

[a] Culture conditions are as in **Fig 2** and **Table II**.
[b] Data are expressed as weight percent of total (mean ± SD; n = 3).

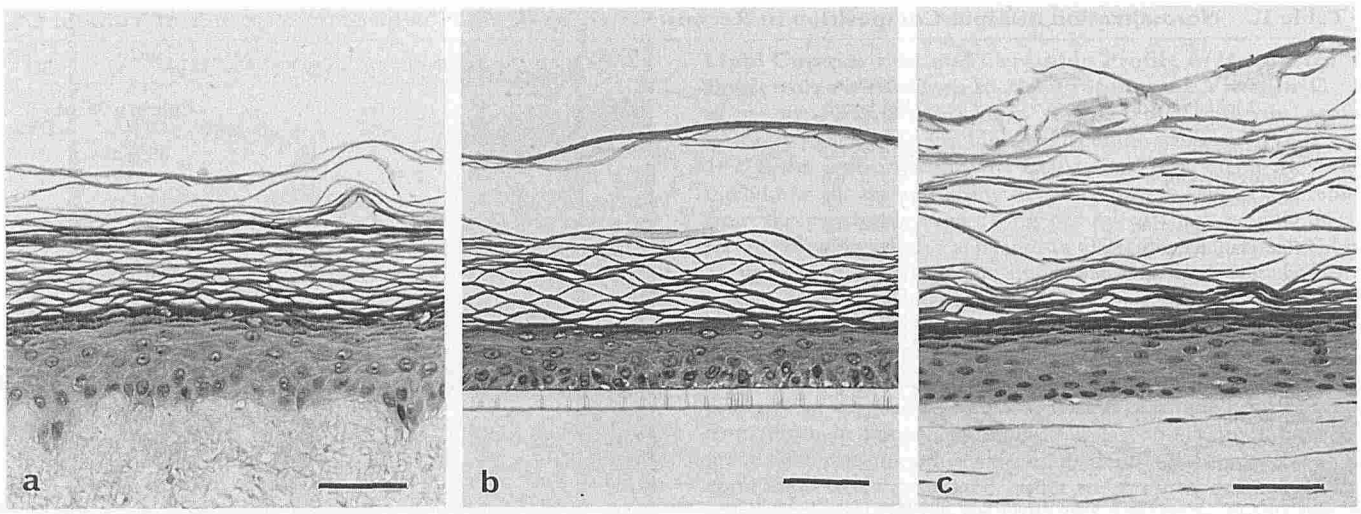

Figure 2. Morphology of epidermis reconstructed on *(a)* DED, *(b)* inert filter, or *(c)* fibroblast-populated collagen matrix. Hematoxylin/eosin staining of paraffin-embedded cultures of keratinocytes grown for 14 d at the air-liquid interface at 37°C in serum-free medium supplemented with vitamin C and E. *Scale bars*, 10 μm.

of a great variety of compounds including water resides in the intercellular lipid domains, it is necessary that the structure, organization, and composition of SC lipids in skin substitutes *in vitro* closely mimic that of the native tissue. In this study we show that culturing of keratinocytes at the air-liquid interface in vitamin C- and E-supplemented serum-free medium results in normalization of epidermal ultrastructure. As in native epidermis (Fartasch *et al*, 1993), numerous LBs are present in the SG and excreted at the SG/SC interface. Furthermore, the exocytosis of LB lipids into the intercellular space is also completed at the SG/SC interface where the lipid lamellae appear with multiple alternating electron-dense and electron-lucent bands arranged in Landmann units exerting the typical broad-narrow-broad-broad-narrow-broad pattern (Landmann, 1986). The intercellular space of the entire SC is filled abundantly with lipid lamellae and the number of intracellularly located lipid droplets is very low. These observations indicate a marked improvement over earlier reported results (Fartasch and Ponec, 1994) with RE-DED generated in serum-containing medium in the absence of vitamins. In those culture systems, an abnormal LB delivery system, disturbance of transformation into lamellar lipid bilayers, and impaired structural organization and distribution of lipids in intercellular spaces were found. Furthermore, the SC showed a compact appearance with abundant lipid droplets within the corneocytes. In the cultures grown in vitamin C- and E-supplemented serum-free medium, not only the SC lipid organization but also the overall SC architecture improved; the SC was less compact and the individual corneocyte layers were loosely bound.

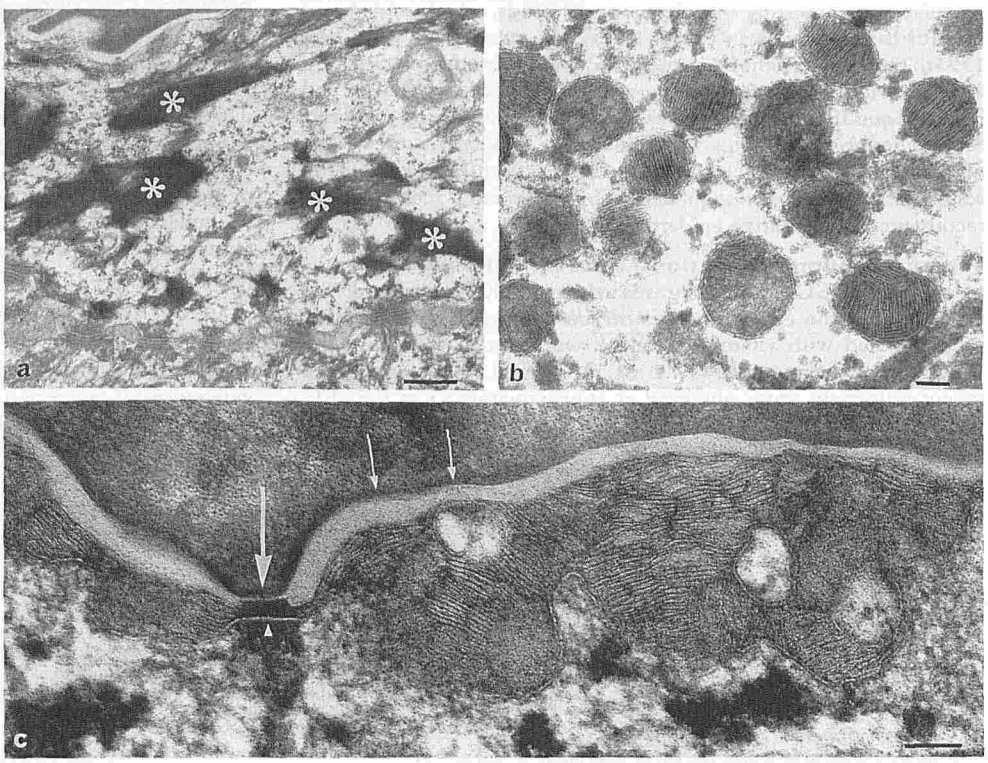

Figure 3. Vitamin C and E supplementation improves the stratum granulosum and stratum corneum ultrastructure in reconstructed epidermis. Transmission electron micrographs showing *(a)* the stellate appearance of keratohyalin granules (*) in the SG; *scale bar*, 0.5 μm; *(b)* the presence of numerous lamellar bodies in the SG; *scale bar*, 0.1 μm; *(c)* the extrusion of lamellar bodies at the SG/SC interface. The periphery of the saccular domains seems to be connected to adjacent desmosomal plugs (*large arrow*). Notice the presence of the cornified envelope (➤) surrounding the desmosomal plug and the roof of the corneocytes. In the uppermost part of the saccular domains, a typical multilayer of lipid lamellae is already formed (*small arrows*); *scale bar*, 0.1 μm. The reconstructed epidermis was generated on DED *(a,b)* or on fibroblast-populated collagen matrix *(c)* either at 37°C *(a,c)* or 33°C *(b)*.

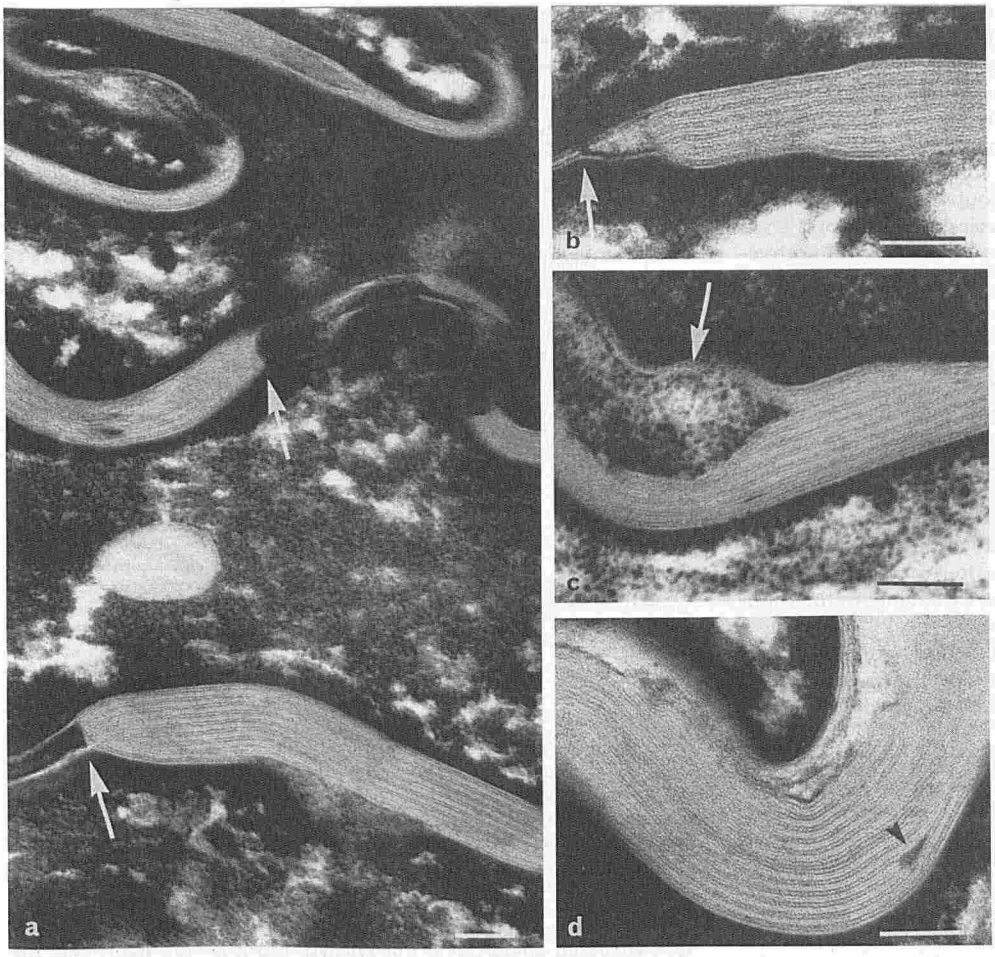

Figure 4. Presence of mature lipid lamellae in the interconeocyte space in reconstructed epidermis. Keratinocytes were cultured in vitamin C- and E-supplemented serum-free medium using DED *(a,c,d)* or inert filter *(b)* as substrates either at 33°C *(a,c)* or37°C *(b,d)*. The multilayered lamellar lipid structures show the characteristic alternating electron-dense and electron-lucent pattern with repeating pattern, as visualized by transmission electron microscopy with ruthenium tetroxide staining. *(a)* Electron micrograph showing that all intercorneocyte spaces are filled with multilayered lamellar lipid structures. Note the round appearance of the edges of the desmosomal plugs (→); *(b)* a desmosomal plug showing pointing appearance (→); *(c)* degradation of desmosomal plugs with diminution of opacity and swelling of their middle part (→); *(d)* remnant of a desmosomal plug with pointed edges surrounded with multilayered lipid lamellar structure in the upper SC layers (➤). *Scale bars*, 0.1 μm.

As *in vivo*, the transformation of desmosomes into corneosomes took place at the SG/SC interface, and the corneosomes became progressively degraded in the upper SC layers. Also, the frequency of corneosomes (percentage of corneocyte membrane length occupied by corneosomes) in the lower SC layers was close to that in native epidermis (20–25%). This is in contrast to results with RE-DED generated in serum-containing medium (Vičanová *et al*, 1996), in which the corneosome frequency was much higher (up to 55%).

In spite of the fact that the corneosome frequency was now similar to that *in vivo*, the desquamation process was still dysregulated, because upon the prolongation of the culture time, the SC thickness gradually increased (data not shown), indicating that epidermal homeostasis has not been reached. It remains to be established whether this can be ascribed to the lack of desquamation *in vitro* or to a declining content of glucosylceramides, but not of ceramides, upon long-term cultivation (data not shown). Glucosylceramides may play an essential role in the regulation of epidermal homeostasis, as recently reported by Marsh *et al* (1995). These authors suggested that due to their opposing effects—glucosylceramides stimulating cell proliferation and ceramides inhibiting proliferation and inducing differentiation—the content of each of these compounds may regulate optimal growth in the epidermis.

The improvement of the SC lipid ultrastructure can most probably be ascribed to a marked improvement of the lipid profile. Inspection of epidermal lipid composition of various available skin substitutes revealed that they differ from that in the native epidermis. The most prominent differences were the low GSL and ceramide contents, especially very low contents of ceramides 6 and 7. The finding that medium supplementation with vitamin C leads to the normalization of both the GSL and the ceramide profiles indicates a key role for vitamin C in the regulation of competent barrier lipids. From our observation it can be further concluded that most of the currently available skin substitutes are generated under vitamin C-deficient conditions. From the current study it also became clear that the substrate on which the keratinocytes are cultured does not affect lipogenesis, because the epidermal lipid composition, LB delivery system, SC lipid organization, and structure were similar irrespective of whether an inert or natural substrate that was populated or not with fibroblasts was used. Also, culturing of keratinocytes at lower temperature (33°C) did not interfere with lipogenesis (data not shown). The finding that no improvement in epidermal lipid profile can be achieved in keratinocyte cultures grown in the presence of vitamin E indicates that the primary role of vitamin C can most probably be ascribed to vitamin C-induced facilitation of hydroxylation of sphingoid bases and of fatty acids and not to its anti-oxidative activity in preventing the formation of free oxygen radicals or lipid peroxides.

It should be noted that the ceramide profile established in this study is based on the mobility of individual ceramide fractions on standard high-performance thin-layer chromatography silica gel. With this approach seven different ceramide fractions in native and reconstructed epidermis have been identified with mobilities comparable to those of pig epidermal ceramides. The total number of free hydroxy groups and the chain length of amide-linked and ester-linked fatty acid govern chromatographic mobility. The same holds true for GSL, where the mobility is further affected by the presence of free hydroxyl groups of glucose. With the thin layer chromatography approach, it is impossible to establish the molecular structure of individual lipids. Therefore, the profile of the sphingoid bases and of the fatty acids in individual (glucosyl)ceramide fractions still remains to be established. This information will also help to elucidate the differences in SAXD profiles observed between native and reconstructed epidermis. This finding was

unexpected, because in both tissues great similarities in lipid lamellar structures were observed by electron microscopy. Although in reconstructed epidermis only a long-range ordering of SC lipids with approximately 12-nm periodicity is observed, in native SC two lamellar phases with periodicities of 6.4 and 13.4 nm are found (Bouwstra *et al*, 1991). In a previous study, only one 6-nm weak peak was found in diffraction curves obtained with SC isolated from RE-DED grown in serum-containing medium, indicating the presence of a long-distance periodicity unit of 12 nm (Bouwstra *et al*, 1995). In this study we found a marked improvement of the SAXD pattern showing the presence of four strong peaks corresponding to the first, second, third, and fourth order, reflecting the presence of well-ordered 12-nm lamellar phase. At present, however, it is not clear why the spacing of the long repeat unit differs from that of the native SC (13.4 nm). One can speculate that the differences in fatty acid length may be responsible, because the repeat distance is expected to be smaller when short-length fatty acids prevail. In addition, it should be noted that the differences in the periodicity of the long repeat unit may also be explained by the accuracy of the method used for this determination. Namely, with native SC the periodicity of the long repeat unit is mostly estimated from curves obtained after recrystallization of SC lipids (by heating SC to 120°C followed by cooling to the room temperature) that contain well-defined sharp peaks reflecting a periodicity of 13.0–13.4 nm. The x-ray diffraction pattern obtained at room temperature contains very broad peaks that make the calculations of the two periodicities with data obtained with native SC at room temperature less accurate.

Most surprising is the finding that the short repeat distance (6.4 nm) is absent in reconstructed epidermis. It remains to be elucidated whether this may be due to the lack of the pH, calcium, or cholesterol sulfate gradients, to slight differences in the relative amounts of individual ceramides, or to differences in the composition of protein-bound hydroxyceramides. Whether or not pH, calcium, or cholesterol sulfate gradients exist in the SC of the reconstructed epidermis is not known, but it is very likely that the presence of these gradients is required for proper SC lipid organization. Namely, in a previous SAXD study, we found that reflections corresponding to the short repeat unit disappeared in recrystallized SC lipids (Bouwstra *et al*, 1991), probably due to disappearance of ionic and lipid gradients caused by mixing of SC lipids during this recrystallization process.

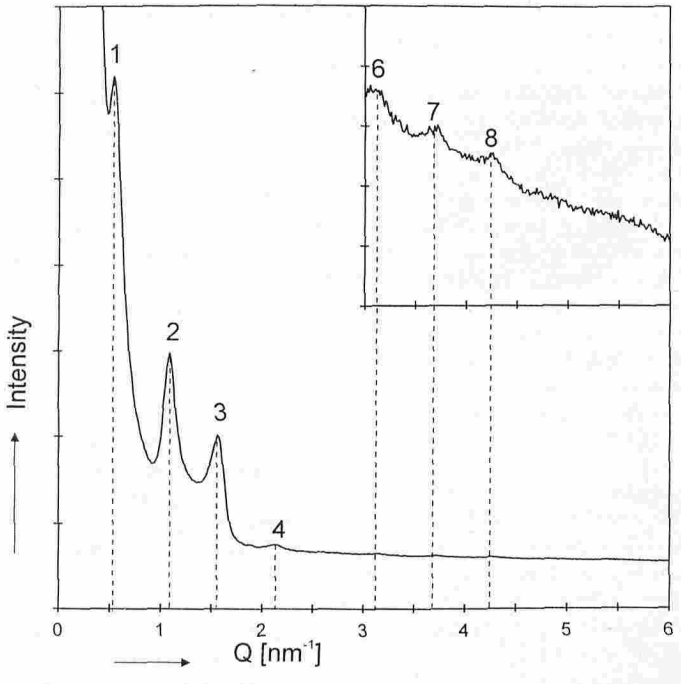

Figure 5. SAXD profile of SC isolated from epidermis reconstructed. The epidermis was generated by seeding keratinocytes on an inert filter and culturing them at 37°C in serum-free medium supplemented with vitamine C and E. *Inset*, The scale for the intensity is different to show the weak intensity peaks. The values denote the spacings of the various orders of the 12-nm lamellar phase.

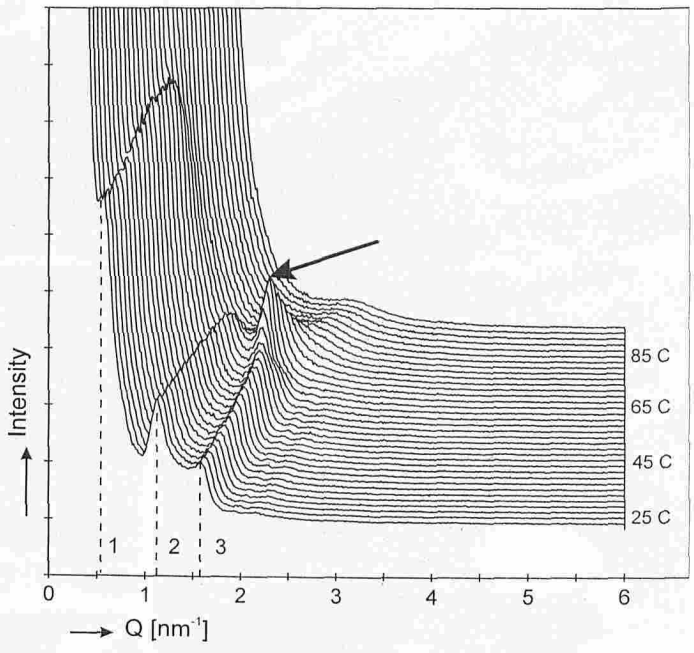

Figure 6. The temperature-induced changes in SAXD profile in SC isolated from reconstructed epidermis. The epidermis was generated by seeding keratinocytes on DED and cultured at 33°C in serum-free medium supplemented with vitamin C and E. Temperatures are indicated in the figure. In each sequential curve the temperature was raised by 2°C. The amounts of material available for the measurements were limited, which resulted in a decrease in the signal-noise ratio in the x-ray curve. The → shows the appearance of the 4.6-nm peak.

The lipid envelope consisting of protein-bound hydroxyceramides has been postulated by Swartzendruber *et al* (1987, 1989) to serve as a template for the proper assembly of SC lipid lamellar structures and to be at least partly responsible for the SC lipid organization. Furthermore, these authors suggest that final SC lipid organization is governed by the interdigitation of lipid envelopes with nonbound interstitial lamellar lipids. The importance of covalently bound ceramides for lamellar ordering of SC lipids is unclear, however, because our recent studies with isolated mixtures of ceramides, cholesterol, and fatty acids revealed the presence of two lamellar phases with both long and short repeat distances, even in the absence of the lipid envelope (Bouwstra *et al*, 1996). The presence of the protein-bound ceramides is probably crucial for the proper orientation of lipid lamellae along the surface of the corneocytes.

In addition, it is tempting to speculate that the desquamation of the horny layer is indirectly involved in the regulation of SC lipids organization. Namely, in CSS grafted onto the nude mouse, only the long-distance repeat unit of 12 nm is found in spite of the finding that the SC lipid profile closely mimics that of the native human SC (Víčanová J, Weerheim A, Harriger D, Boyce ST, Ponec M, unpublished results). It should be noted that in grafted CSS the desquamation process is most probably disturbed, because its SC is thicker than in normal human epidermis. In addition, the results of a pilot study from our laboratory (unpublished results) suggest differences in lamellar organization between the lower and the upper SC; in the upper SC layer the short distance repeat unit prevails. Furthermore, interbilayer organization and also desqua-

mation may be affected by calcium bridges between cholesterol sulfate molecules in apposed lipid leaflets, as suggested by Epstein *et al* (1981). At present no information is available as to whether the extracellular calcium distribution is similar *in vivo* and *in vitro*.

The reconstruction of epidermis in the presence of vitamin C results in a marked improvement of SC architecture and lipid profile. The finding that only skin equivalents formed in vitamin C-supplemented medium are capable of synthesizing sufficient amounts of all (glucosyl)ceramide classes indicates the crucial role of vitamin C in the regulation of the synthesis of competent barrier lipids.

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